American Journal of Medical Genetics (Neuropsychiatric Genetics) 67:139–146 (1996)

Analysis of Thirteen Trinucleotide Repeat Loci as Candidate Genes for Schizophrenia and Bipolar Affective Disorder

Sanjeev Jain, Jayne Leggo, Lynne E. DeLisi, Timothy J. Crow, Russell L. Margolis, Shi-Hua Li, Sandy Goodburn, Cathy Walsh, Eugene S. Paykel, Malcolm A. Ferguson-Smith, Christopher A. Ross, and David C. Rubinsztein

East Anglian Regional Genetics Service Molecular Genetics Laboratory (S.J., J.L., M.A.F.-S., D.C.R.) and Departments of Psychiatry (S.J., C.W., E.S.P.) and Clinical Genetics (S.G., M.A.F.-S.), Addenbrooke's NHS Trust, Cambridge, United Kingdom; National Institute of Mental Health and Neurosciences (S.J.), Bangalore, India; Laboratory of Molecular Neurobiology, Departments of Psychiatry and Neuroscience (R.L.M., S.-H.L., C.A.R.), Johns Hopkins Medical School, Baltimore, Maryland; Department of Psychiatry (T.J.C.), University of Oxford, Warneford Hospital, Oxford, United Kingdom; Department of Psychiatry and Behavioural Science, State University of New York at Stony Brook (L.E.D.), Stony Brook, New York; and Department of Pathology (M.A.F.-S.), Cambridge University, Cambridge, United Kingdom

A group of diseases are due to abnormal expansions of trinucleotide repeats. These diseases all affect the nervous system. In addition, they manifest the phenomenon of anticipation, in which the disease tends to present at an earlier age or with greater severity in successive generations. Many additional genes with trinucleotide repeats are believed to be expressed in the human brain. As anticipation has been reported in schizophrenia and bipolar affective disorder, we have examined allele distributions of 13 trinucleotide repeat-containing genes, many novel and all expressed in the brain, in genomic DNA from schizophrenic (n = 20–97) and bipolar affective disorder patients (23–30) and controls (n = 43–146). No evidence was obtained to implicate expanded alleles in these 13 genes as causal factors in these diseases. © 1996 Wiley-Liss, Inc.

KEY WORDS: schizophrenia, bipolar affective disorder, trinucleotide repeats

INTRODUCTION

Recently there has been an increasing interest in a new class of genetic diseases caused by abnormal expansions of tracts of trinucleotide repeats. These diseases (that include fragile X, myotonic dystrophy, and Huntington's disease [HD]) all affect the nervous system and share a number of interesting features. First, although normal chromosomes are polymorphic with respect to repeat length, they show very low mutation rates. However, mutant chromosomes with long repeats are highly mutable and tend to increase their repeat number in successive generations. Second, as a general rule, increasing disease severity and/or decreasing age of onset of symptoms correlate with increasing size of the triplet expansions. These molecular features can explain the phenomenon of anticipation, which we understand today as the tendency for the disease to manifest at an earlier age in successive generations [Willems, 1994].

There have been reports of anticipation in mental illness including the initial paper of F.W. Mott [1911] in which he coined the term, "the law of anticipation of the insane." Recent reports have suggested that anticipation may be a feature of both schizophrenia and bipolar affective disorder [e.g., Ross et al., 1993; Basset and Honer, 1994; McInnis et al., 1993, personal communication].

Although there is convincing evidence that supports a major genetic contribution to the etiology of schizophrenia and manic depressive illness, the mode of inheritance and the number of genes involved in each case are not known [Goldin et al., 1992; Hanson and Gottesman, 1992]. Accordingly, we have investigated 11 trinucleotide repeat-containing genes expressed in the brain (which are normally polymorphic in repeat length) as candidate genes for these 2 disorders. In addition, we have included the CAG repeats in the HD and dentatorubral-pallidoluysian atrophy (DRPLA or CTG B37) genes as candidates for bipolar affective disorder, as depression may be a primary consequence of the neuropathological changes in HD [Morris, 1991]

Received for publication March 2, 1995; revision received May 16, 1995.

Address reprint requests to David C. Rubinsztein, East Anglian Regional Genetics Service Molecular Genetics Laboratory, Box 158, Addenbrooke's NHS Trust, Hills Road, Cambridge, CB2 2QQ, UK.

since it often precedes other symptoms, and as psychotic features have been described in patients with DRPLA [Koide et al., 1994]. Both of these loci have been previously found to be normal in most schizophrenic patients [Rubinsztein et al., 1994b,c; St. Clair, 1994].

Two isolated cases of schizophrenia out of more than 300 patients examined had 36 CAG repeats in the HD gene [Rubinsztein et al., 1994c; St. Clair, 1994]. The smallest HD alleles described have 36 repeats. However, we have identified normal individuals with 36 HD repeats as well, and it is therefore impossible to determine the pathological significance of the 36 repeats in these schizophrenic patients [Rubinsztein et al., 1994c].

MATERIALS AND METHODS

Patients

Ethical approval has been granted for the analysis of candidate genes in patients with schizophrenia and bipolar affective disorder.

Schizophrenic Patients

Families in which there were at least 2 sibs with either schizophrenia or schizoaffective disorder were identified from 3 main sources: 1) A cohort of cases receiving clinical care in north west London and its surrounding regions and other cases associated with the National Schizophrenia Fellowship; 2) a US national registry of families identified from treatment centers within a New York county catchment area (Suffolk county), through clinician referrals, and advertisements throughout the United States, particularly with the aid of the National Alliance for the Mentally Ill; 3) families recruited in a similar manner by psychiatrists from the Institute of Psychiatry of the University of Milan, Italy, from surrounding northern Italian communities. Blood samples were collected and DNA was prepared from these predominantly nuclear families. Only unrelated schizophrenic probands were investigated, except when we needed to perform family studies in order to determine whether an expanded allele cosegregated with the disease. All persons were evaluated by a trained clinician. Subjects were interviewed using a modified Schedule for Affective Disorders and Schizophrenia (SADS) structured format [Endicott and Spitzer, 1978]. Records from previous admissions to hospitals and of psychiatric treatment were obtained, and further information was collected on each person from reliable family members. Diagnoses were made based on information from these multiple sources using DSM-III-R criteria for major psychiatric and schizophrenia spectrum personality disorders. A core group of 24 schizophrenic patients randomly chosen from the 99 probands we had available in the Cambridge laboratory was used to analyze the majority of the loci (22 males, 2 females; 17 from the United States, 6 from the United Kingdom, 1 from Italy). The precise number of schizophrenic patients examined at each locus can be found in Table I.

Bipolar Affective Disorder

Patients were recruited from inpatient and outpatient clinics at a hospital in East Anglia. The sample met Research Diagnostic Criteria [Spitzer et al., 1978] for bipolar affective disorder I. All were of English Caucasian origin and between 19 and 65 years of age. Lifetime psychopathology was assessed by trained clinicians using the SADS-Lifetime Version (SADS-L) interview [Endicott and Spitzer, 1978] supplemented by case note review. All cases were unrelated. A core group of 24 patients with bipolar affective disorder (11 males and 13 females) were used to analyze the majority of the loci examined. Nine of these patients had no family history of psychiatric illness; 8 patients had a family history of affective disorder (bipolar affective disorder or major depression) in a first-degree relative; 4 patients had second- or third-degree relatives with a history of affective disorder (bipolar affective disorder or major depression); 3 patients had first- or second-degree relatives who had a history of schizophrenia. The precise number of patients examined at each locus can be found in Table I.

Controls

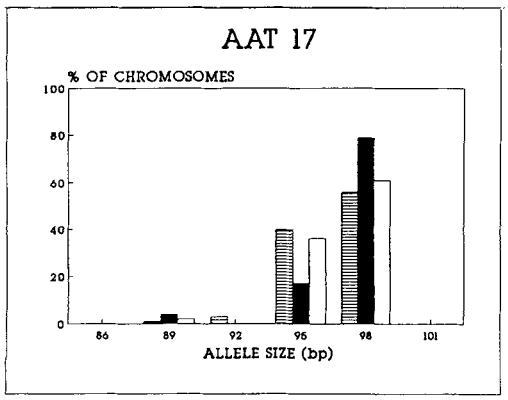
Previous experience of the 8 trinucleotide repeat diseases that have been characterized suggests that the normal and disease size ranges are generally distinct [Sutherland and Richards, 1993]. (The cases of normal individuals with 36 HD repeats represent an exception to this rule. Such cases would seem to be extremely rare and have a prevalence of less than 1/1,000 if one combines data in Rubinsztein et al., 1994a,c; St. Clair, 1994.) Thus, if bipolar affective disorder and schizophrenia are associated with trinucleotide repeat expansions that follow the precedents set by the diseases that have already been characterized, one would expect that the mutant alleles would be clearly larger than the largest normal alleles.

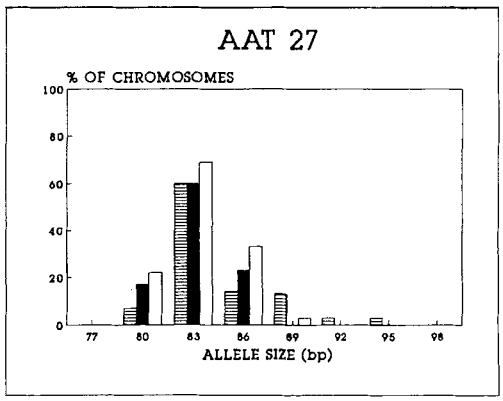
We examined anonymized, unrelated, control samples from a variety of ethnic groups [populations described in detail in Rubinsztein et al., 1994a]. The East Anglians were always used as controls as they were a good match with the bipolar patients (who were East Anglian) and a reasonable ethnic match with most of the schizophrenic patients. However, we were aware that it was unlikely that an East Anglian sample would fully represent the normal range of allelic diversity at these loci—studies of the CAG and CCG repeats in the HD gene and analyses of microsatellite loci suggest that normal allelic diversity at short tandem repeat loci is greater in African populations [Rubinsztein et al., 1994a; Bowcock et al., 1994]. Therefore we included Sub-Saharan African controls (mainly from Nigeria) to increase the range of normal alleles and thus avoid possible false-positive results that may have arisen from finding a rare large normal allele. For loci GLU R6 and CTG B33, where we detected alleles in the patient groups that were larger than what we had observed in the East Anglian and African controls, we also examined Asian Indians and South African Blacks to further increase the allelic diversity. Controls were not screened by psychiatrists to exclude subtle signs of illness (see Results section for power calculations, which take this into account). Details of the numbers of patients and controls that were screened for the various loci are shown in Table I.

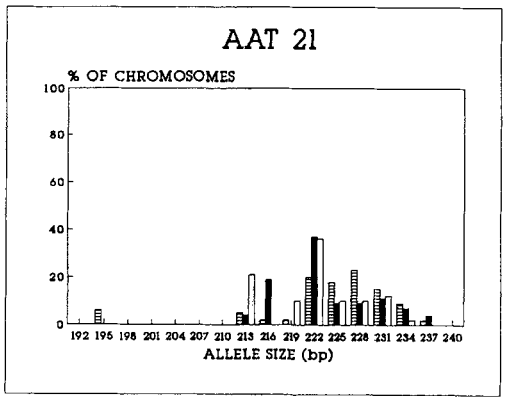
TABLE I. Loci, Primer Sequences, Annealing Temperature, and Number of Subjects Studied for Each Marker*

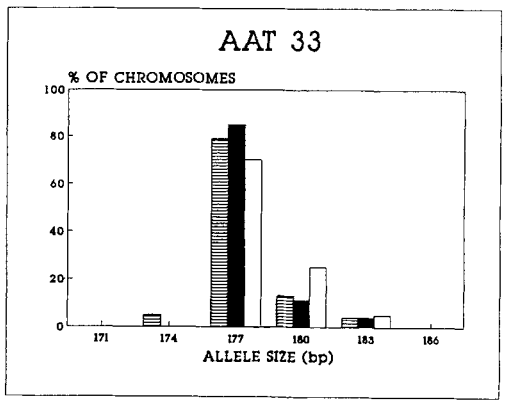
Locus	Chromosome	Primer sequences	A.T. (°C)	Number of individuals analyzed in patients and controls						
				EA	BA	AI	SAB	J	S	BP
AAT21 $(AAT)_n$	6	5′-GTCCTCAGTAACCAATG 5′-AGAGCTGCCAGAAGGTGA	55	22	22	—	—	—	21	23
CCA38 $(CCA)_n$	5	5′-TGGCCCTTCATTTTCTAACT 5′-TGGCTGTAAGACTGAGAGGA	60	24	23	—	—	—	23	24
AAT27 $(AAT)_n$	?	5′-CCTGGTCAAAGGTTTATTAT 5′-TTATTGGAACTCACATTTTA	46x3 44x3 42x30	22	23	—	—	—	23	24
GLU R6[a] $(TAA)_n$	6	5′-CAACACCTTTTCTCTAACCCC 5′-CTCGGCCAGTTTTTACAACTTG	55	24	21	25	23	—	22	24
CAG26a $(CAG)_n$	?	5′-CCGCGGCAAGAGCCAGCAGC 5′-TTCTCATCTTTCTCCTCCTG	58x3 56x3 54x33	24	31	—	—	—	24	24
AAT47 $(AAT)_n$	12	5′-ATGCTGTTGTTTAGGGGTAA 5′-AACGTCAGCTCCTTTATTAT	45x3 43x3 41x30	24	22	—	—	—	23	24
AAT40 $(AAT)_n$	10	5′-TGCATTTATAGATTTGATGAC 5′-GATGGAGCAAGAGCCTGTCTC	55	24	22	—	—	—	20	24
DRPLA[b] $(CAG)_n$	12	5′-CACCAGTCTCAACACATCACCATC 5′-CCTCCACTGGGTGGGGAAATGCTC	64	—	—	—	—	146	—	24
AAT42 $(AAT)_n$	2	5′-GGGCAGCAGAACGAAACTCT 5′-CCTAAAAGGGGACCATTGTT	46	23	23	—	—	—	23	23
AAT33 $(AAT)_n$	12	5′-AATAATTCCTCCTCCTG 5′-GCTACCGTATTCCATTT	49x2 47x2 45x33	23	20	—	—	—	20	23
HD-CAG[c] $(CAG)_n$	4	5′-ATGAAGGCCTTCGAGTCCCTCAAGTCCTTC 5′-GGCGGTGGCGGCTGTTGCTGCTGCTGCTGC	65	40						30
CTG B33[b,d] $(CTG)_n$	3 (12?)	5′-CAAAAAAGCACCTGGTATAA 5′-GGGCTGGAGCCTTTTTACTCGC	60	53	20	33	32	—	97	24
AAT17 $(AAT)_n$	22	5′-TCAACTTTTGGCTTTGTTTTT 5′-CCTGGGCAATATGGCAAGATA	49x2 47x2 45x33	24	23	—	—	—	22	24

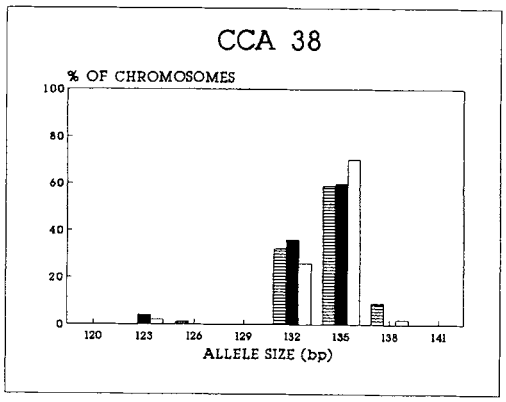
* Primer sequences for all loci except the following are from Margolis et al., 1995 (AAT loci that are not lettered) and Margolis et al., in preparation (other unlettered loci). [a] Paschen et al. [1994]; [b] Koide et al. [1994] for normal Japanese sizes [primers described by Li et al., 1993]; [c] Warner et al. [1993]; [d] Li et al. [1993]. DRPLA is also known as CTG-B37.

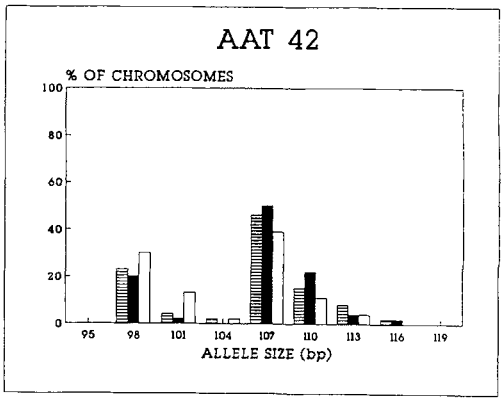

AAT 17
% OF CHROMOSOMES
100
80
60
40
20
0
86
89
92
95
98
101
ALLELE SIZE (bp)

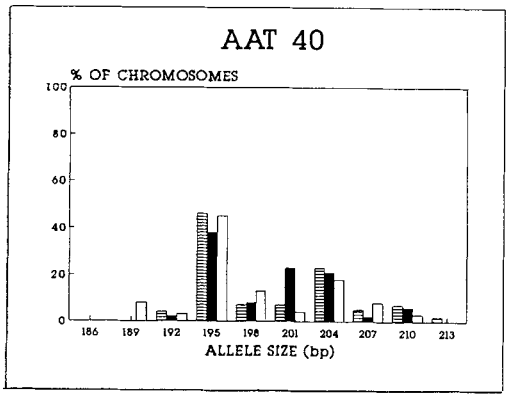

AAT 27
% OF CHROMOSOMES
100
80
60
40
20
0
77
80
83
86
89
92
95
98
ALLELE SIZE (bp)

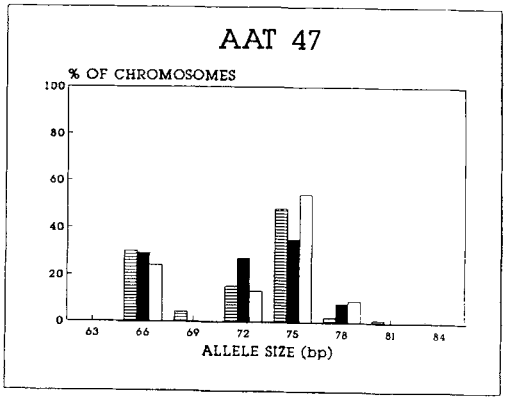

AAT 21
% OF CHROMOSOMES
100
80
60
40
20
0
192 195 198 201 204 207 210 213 216 219 222 225 228 231 234 237 240
ALLELE SIZE (bp)


AAT 33
% OF CHROMOSOMES
100
80
60
40
20
0
171
174
177
180
183
186
ALLELE SIZE (bp)


CCA 38
% OF CHROMOSOMES
100
80
60
40
20
0
120
123
126
129
132
135
138
141
ALLELE SIZE (bp)


AAT 42
% OF CHROMOSOMES
100
80
60
40
20
0
95
98
101
104
107
110
113
116
119
ALLELE SIZE (bp)


AAT 40
% OF CHROMOSOMES
100
80
60
40
20
0
186
189
192
195
198
201
204
207
210
213
ALLELE SIZE (bp)


AAT 47
% OF CHROMOSOMES
100
80
60
40
20
0
63
66
69
72
75
78
81
84
ALLELE SIZE (bp)

A.

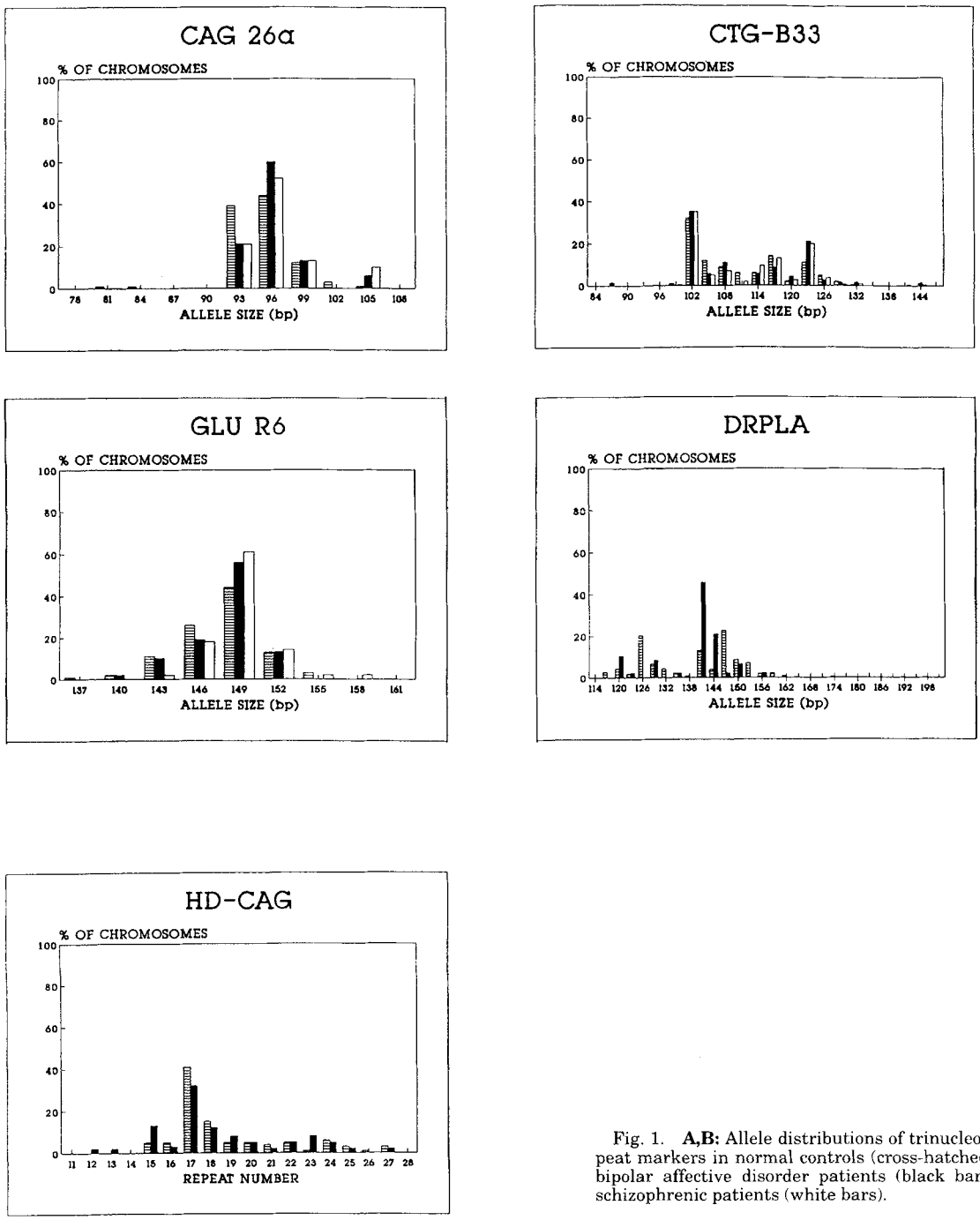


Fig. 1. **A,B:** Allele distributions of trinucleotide repeat markers in normal controls (cross-hatched bars), bipolar affective disorder patients (black bars), and schizophrenic patients (white bars).

Polymerase Chain Reaction (PCR) Conditions and Loci Examined

In addition to the HD [Warner et al., 1993] and DRPLA CAG repeats [Li et al., 1993; Koide et al., 1994], we examined 11 polymorphic trinucleotide repeat-containing loci from genes expressed in the brain. These were identified using oligonucleotide repeat probes to screen cDNA libraries as previously described [Li et al., 1993; Margolis et al., 1995]. PCR reactions were labeled either with α^{32}P-dCTP or with an end-labeled primer and the PCR products were compared to a known sequence ladder on denaturing acrylamide gels. In all cases a control sample was run on different gels for each locus in order to confirm the relative sizes of alleles. Table I shows the primer sequences, annealing temperatures, and the number of chromosomes analyzed in each group.

RESULTS

Allele sizes in patients and controls are shown for the different loci in Figure 1. In all cases except for loci GLU R6 and CTG B33, the repeat sizes in the patients

were within the normal size range. For GLU R6, one schizophrenic patient had an allele (158 bp = 27 repeats) that was one repeat larger than the largest observed normal allele (93 controls were investigated). However, the schizophrenic sib of this patient did not have the 158 bp allele. This suggests that the GLU R6 158 bp allele is not pathogenic in this family. CTG B33 was a more difficult repeat to assess as the PCR primers detect loci on chromosomes 3 and 12 [Li et al., 1993]. Consequently, up to 4 alleles per individual can be detected with these PCR primers. One schizophrenic patient and one bipolar affective disorder patient had alleles of 22 repeats that were one repeat outside the normal range (138 controls were examined). Schizophrenia did not segregate with the large allele in the schizophrenic patient's family: Although the proband had the large allele, it was not present in one of his affected sibs but was present in one of his unaffected sibs. An additional reason why these CTG B33 and GLU R6 alleles are unlikely to be causing disease is that they both have far fewer repeats than the smallest alleles (36 repeats) that have been described on mutant chromosomes for any of the trinucleotide repeat diseases [Sutherland and Richards, 1993; Rubinsztein et al., 1994b]. It is likely that these alleles are merely comparatively rare larger normal alleles.

Power statistics were performed to determine the likelihood of detection of expanded alleles using the sample sizes in this study. A number of assumptions were made when constructing the models that were analyzed. First, studies of the 8 described trinucleotide repeat diseases suggest that there are distinct normal and disease allele size ranges with practically no overlap [Sutherland and Richards, 1993]. The only cases where overlap has possibly been described are in HD, where the prevalence of both normal and mutant alleles with 36 CAG repeats is much less than 1% [Rubinsztein et al., 1994a,c; St. Clair, 1994; Kremer et al., 1994]. Second, we have made the conservative assumption that the frequency of individuals in the general population who carry schizophrenia or bipolar affective disorder susceptibility genes is 1/50. (Thus we assume that 1/50 controls will carry susceptibility genes.)

We have tested a set of scenarios where we compare 20 patients with 20 controls. The scenarios consider varying degrees of genetic heterogeneity in schizophrenia/bipolar affective disorder where the proportion of patients who carry the specific putative disease-associated trinucleotide expansion that is being examined varies from 100 to 5% (equivalent to P1 in Table II). We have assumed that this proportion is the same in the patient and control groups. For instance, if only 50% of schizophrenia is caused by a particular trinucleotide repeat expansion, then this expansion will be expected to be found in 1% of controls (50% of 1/50; P2 in Table II). Thus, the relative risk of finding a trinucleotide repeat expansion in the patient group compared to the controls in this model is 50, irrespective of the proportion of schizophrenia/bipolar affective disorder that is attributable to the locus.

A 2-sided binomial test of proportions was performed with the significance level (α) set at 0.05 assuming equal sample sizes of 20 in the case and control groups [Table II; Rosner, 1990]. The probability of detecting a significant excess of large expansions in the cases was greater than 80% for all the scenarios where the proportion of cases that were associated with expansions exceeded 33% (Table II).

DISCUSSION

Both schizophrenia and bipolar affective disorder show features that are compatible with anticipation [Basset and Honer, 1994; McInnis et al., 1993; Ross et al., 1993; McInnis et al., personal communication]. These studies examined sets of families with schizophrenia and bipolar affective disorder which were not preselected for having anticipation. If anticipation is truly a feature of these diseases, it is likely to be a strong and fairly generalized effect, as it would not be detected if it were only present in a small minority of cases. Accordingly, we have investigated a set of genes expressed in the brain that contain polymorphic trinucleotide repeats as possible candidates for these disorders. Our results suggest that abnormal expansions of the trinucleotide repeats in the specific genes that we

TABLE II. Power Statistics for Varying Models of Disease Inheritance*

No. cases	Proportion of cases associated with expansions (P1)	No. controls	Proportion of controls associated with expansions (P2)	Power (%)
20	1.00	20	.02	>99
20	.50	20	.01	97
20	.33	20	.0066	80
20	.20	20	.004	61
20	.10	20	.002	30
20	.05	20	.001	16

* The scenarios consider varying degrees of genetic heterogeneity in schizophrenia/bipolar affective disorder where the proportion of patients who carry the specific putative disease-associated trinucleotide expansion that is being examined varies from 100 to 5% (equivalent to P1). We have assumed that this proportion is the same in the patient and control groups. For instance, if only 50% of schizophrenia is caused by a particular trinucleotide repeat expansion, then the expansion will be expected to be found in 1% of controls (50% of 1/50) (P2). A 2-sided binomial test of proportions was performed with the significance level set at 0.05 assuming equal sample sizes of 20 in the case and control groups [Rosner, 1990].

have examined are unlikely to be major etiological factors for schizophrenia or bipolar affective disorder.

However, a few caveats should be considered. First, the inheritance of both bipolar affective disorder and schizophrenia is unclear [Goldin et al., 1992; Hanson and Gottesman, 1992]. It is difficult to absolutely exclude candidate genes in disorders with variable penetrance that may be polygenic, as "mutant" alleles may be found in normal controls. Furthermore, it is possible that the prevalence of the putative trinucleotide repeat expansions may be different in familial cases relative to isolated cases. These possibilities are included in the modeling that was used for the power statistics that allowed us to deduce that we had a high likelihood of detecting abnormal expansions in situations where the putative trinucleotide repeat expansion was associated with 33% or more of the disease cases (see Table II). These power statistics were performed with sample sizes of 20 cases and 20 controls.

Second, one may argue that we have not detected putative expanded repeats as these would have been resistant to PCR amplification. A number of precautions were taken to try to address this possibility. First, gels were exposed to X Ray film for longer than necessary for detection of normal-sized products, so that any larger, fainter PCR products would have a better chance of being detected. Second, heterozygosity indices were determined for the control and patient groups. In a situation where large repeats were resistant to amplification (e.g., fragile X), one would expect to see that the heterozygosities of the affected group(s) would be very low compared to the controls. However, we did not find this result. The heterozygosities of all of the markers were compared to those of the controls using the chi-square test (2 populations) or the chi-square test of independence (3 populations) (data not shown). The only marker that showed a significant difference ($P = 0.03$) was CCA38. The difference was due to the high heterozygosity value of the African population—African populations are known to have higher heterozygosities at microsatellite and trinucleotide repeat loci [Bowcock et al., 1994; Rubinsztein et al., 1994a]. No difference was apparent when the Africans were removed from the control group and the patients' heterozygosity data were compared to the Caucasian controls ($P = 0.2$) (all except 3 of the patients examined for this marker were Caucasian).

In conclusion, our results suggest that none of the trinucleotide repeat loci we have examined are abnormally expanded in most patients with bipolar affective disorder and schizophrenia. The commitment of the search for trinucleotide-containing genes in these disorders depends on the strength of the clinical evidence for anticipation. Such studies can be confounded by ascertainment bias, as pointed out by Penrose [1948] in his work on myotonic dystrophy. Although there was much debate as to the biological significance of the phenomenon of anticipation in myotonic dystrophy, it is instructive to note that this disease became a paradigm for anticipation and unstable trinucleotides after its molecular nature was elucidated [Harper et al., 1992]. Due to the complex genetics of schizophrenia and bipolar affective disorder, we believe that suggestions of anticipation should be seriously considered and pursued by confirmatory clinical studies and by examination of trinucleotide-containing genes as candidates. One approach to this problem would be to perform a genome scan for abnormally expanded trinucleotide repeats using techniques like the repeat expansion detection method (RED) described by Schalling et al. [1993]. However, the original descriptions of this methodology were confined to detection of very large expansions such as those seen in fragile X but the system was not designed to detect smaller expansions like those seen in HD. In this study we have only tested a relatively small proportion of the total number of trinucleotide-containing genes that are believed to exist. About 50–100 highly polymorphic CAG-containing genes are thought to be present in the human genome [Ross et al., 1993]. We have also only tested CAG- and AAT-containing genes. While CAG expansions have been associated with neurodegenerative disorders, there have been no reports of AAT expansions associated with disease [Willems, 1994]. Future loci that will be examined will also include CCG repeats which have been associated with neurodevelopmental disorders like fragile X [Willems, 1994]. We believe that this study demonstrates the feasibility of this approach to detect expanding trinucleotide repeats in these populations.

ACKNOWLEDGMENTS

We thank Drs. Antonio Vita, Carla Morganti, John Old, and Raj Ramesar for patient and control DNA samples and Jay Dann for technical support. This work was supported by the Huntington's Disease Association (D.C.R. and M.A.F.-S.), NIMH-MH50763, The Stanley Foundation and the Scottish Rite Schizophrenia Foundation (C.A.R.), A NARSAD Young Investigator's award and a Clinician Scientist award (R.L.M.), a Commonwealth Fellowship (S.J.), the UK MRC (T.J.C.), and NIMHR01-844245 (L.E.DL.).

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